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The Effect of Warming and Nitrogen Addition on Soil Aggregate Enzyme Activities in a Desert Steppe

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Abstract

Soil enzymes secreted by microorganisms play a key role in carbon (C), nitrogen (N), and phosphorus (P) metabolism in soil organic matter. As major drivers of climate change, warming and nitrogen addition affect soil physicochemical properties and enzyme activity, but their combined effects on these parameters across different soil aggregate size scales in desert steppes remain unclear. This study used a 2 × 2 factorial split-plot design (control; warming; nitrogen addition: warming + nitrogen addition) conducted from 2006 in Inner Mongolia's desert steppe. Soil samples were collected in 2018–2019, and aggregates were fractionated into >2000 μm, 250–2000 μm, and <250 μm sizes using a modified dry-sieving method. Physicochemical properties and enzyme activities were measured. Our results show that warming significantly reduced the total nitrogen (TN) and organic carbon (SOC) content in aggregates, while nitrogen addition significantly decreased the pH value in aggregates but had no significant impact on other soil nutrient content indicators. For soil enzyme activity, warming significantly reduced the activity of Urease and Alkaline Phosphatase (ALP) in soil aggregates, and nitrogen addition significantly reduced the activity of Urease, ALP, and β-glucosidase (BG) in aggregates. However, the size of the aggregates had a significant impact on the activity of Urease and BG. The influence of soil physicochemical properties on different enzyme activities varied across different years. These findings indicate that under the global change scenario, the physicochemical properties and enzyme activity of desert steppe soils are affected by warming and nitrogen addition to varying degrees, and the impact of these two factors shows significant differences across different years. Moreover, the interactive effects of warming and nitrogen addition did not simply result in an additive effect influenced by single factors.

Keywords: desert steppe; climate change; soil aggregates enzyme

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1. Introduction

Human activities like burning fossil fuels and applying chemical fertilizers have triggered global changes, including rising temperatures, altered precipitation patterns, and atmospheric nitrogen addition [1,2]. Among these, climate warming and nitrogen addition are key drivers of soil ecosystem changes [3–5].

Soil extracellular enzymes, predominantly produced by microorganisms, act as the immediate drivers of organic matter breakdown in soils [6]. Enzyme activity reflects the

catalytic process of soil microorganisms continuously acting in response to environmental changes, and can be used to assess microbial nutritional needs and establish an ecological environmental response index [7]. Additionally, soil enzymes can decompose organic nutrients in the soil into available forms directly usable by plants and microorganisms. Released by microorganisms or generated from cell lysis, these enzymes influence soil nutrient conversion and cycling, reflecting dynamic changes in nutrient availability. For example, BG indicates the cycle of carbon in the soil. Disaccharides and trisaccharides are hydrolyzed into small molecules of glucose [8]. Urease plays an important role in the soil nitrogen cycle. It can hydrolyze the urea in the soil to make it an effective nutrient available to plants [9]. Phosphatases, generated by plant roots, mycorrhizal fungi, saprotrophic fungi, or bacteria, display varying pH and temperature optima. Both their synthesis and activity are pivotal in driving soil P cycling [10]. Because the secretion of soil microorganisms is the main source of soil enzymes, and Dehydrogenase (DHA) is mainly present in living cells, so its activity can characterize the overall activity of microorganisms [11]. Soil aggregates, as the basic units of soil structure, provide habitats and ecological niches for microbial communities while promoting material cycling and energy transfer in terrestrial ecosystems. They maintain microbial activities by regulating soil environmental conditions like aeration and nutrient retention, thus driving material exchange and energy flow in ecosystem processes [12,13]. Studies have shown that in small-sized soil aggregates, bacteria are sufficiently protected to avoid predation risks, while fungi, due to their generally larger size compared to bacteria, find it difficult to colonize within small aggregates [14]. Meanwhile, macro-aggregates enrich more microbial abundances associated with SOC than micro-aggregates, exhibit stronger interactions in microbial networks, and contain more functional genes related to carbon and nitrogen metabolic pathways [15]. The stability of soil aggregates is an important physical property of soil, which has a significant influence on soil ventilation, temperature, mechanical resistance, and water and nutrient maintenance [16,17]. The formation and destruction of soil aggregates are usually accompanied by the retention and release of soil nutrients [18]. However, based on current knowledge, studies on the relationships between the contents of C, N, and P associated with soil aggregates and soil enzyme activities under warming and nitrogen addition conditions are limited [19].

In many previous studies, researchers studied the effects of warming and nitrogen addition on the dynamics of soil enzyme activities in soil aggregates of grassland ecosystem, but with inconsistent results so far. Sardans have shown that when the soil temperature increases in summer, the activities of soil Urease and BG are reduced, but in winter the activity of soil enzymes increase with warming [20]. Higher soil temperatures in semi-arid temperate grassland regions reduce soil BG activity [21]. At the same time, however, studies have also shown that warming can promote the BG activity in the soil of the Mediterranean natural bush forest [20]. Studies in the Qinghai-Tibet Plateau have shown that increasing temperature can increase soil Phosphatase activity [22], while another study in Wencai Grassland showed that warming had no significant effect on Phosphatase activity [23]. Many studies have explored the effects of nitrogen addition on bulk soil enzyme activity and related mechanisms, with results varying across ecosystems. A study on British pastures and grasslands found that nitrogen addition had no significant effect on soil BG activity [24]. However, other studies have shown that nitrogen addition can promote soil BG activity. At the same time, nitrogen addition can also promotes the activity of Phosphatase [25]. Studies on typical grasslands in Inner Mongolia showed that nitrogen addition significantly reduced the activities of soil DHA, AP and BG [26]. The distribution characteristics of enzyme activity aggregates play a vital role in the stability of the organic matter of the aggregates [27], but the research on the influence of changes in the external environment on

the distribution of microbial enzyme activities in soil aggregates is relatively lacking. Micro aggregates support a more diverse microbial community and higher enzyme activity than large aggregates [28,29]. Studies have shown that carbohydrate hydrolase (such as BG and cellulase) tends to be distributed in large aggregates, while the distribution characteristics of leucine trypticase and phosphatase show the opposite pattern [30]. However, studies have also found that carbohydrate hydrolases (such as BG and cellulase) have higher activity in micro-aggregates [31]. From this, we can surmise that the distribution characteristics of microbial enzyme activity in soil aggregates varies with external environmental factors.

Previous studies have mostly focused on the effects of single factors such as warming or nitrogen addition on soil physicochemical properties and enzyme activities, and most of them were conducted on bulk soil. In contrast, research on the interactive effects of these two factors at the soil aggregate level is relatively limited. Therefore, in this study of *Stipa breviflora*—a constructive species of Inner Mongolian desert grassland—we used an experimental platform of simulated warming and nitrogen addition for 14 years to evaluate the size of soil aggregates and to study the effects of warming and nitrogen input on the distribution and activity of C-, N- and P-acquiring enzymes. We hypothesized that: (1) N addition stimulates C and P acquisition enzymes because higher soil N availability may lead to C and P restriction; and (2) The effects of warming and nitrogen addition on the particle size distribution of soil aggregates, the contents of C, N, P pools, and soil enzyme activities vary with the size of soil aggregates. By linking aggregate-scale processes to global change drivers, this study provides critical parameters for improving ecosystem models and guiding sustainable land management in arid agro-ecosystems, where maintaining soil structure and nutrient cycling is essential for agricultural resilience against climate change.

2. Materials and Methods

2.1. Field Site and Experimental Design

The study area is located at the Comprehensive Experimental Demonstration Center (111°53′ E, 41°46′ N) of the Academy of Agriculture and Animal Husbandry in the mid-southern Siziwang Banner, Ulanchabu City, Inner Mongolia Autonomous Region, China. It belongs to a typical mid-temperate continental monsoon climate zone, characterized by long and cold winters, sparse but concentrated summer precipitation, and large inter-annual temperature differences. The experimental area has relatively few plant species, with a low and sparsely distributed grass layer. The grassland type is *Stipa breviflora* + *Artemisia frigida* + *Cryptocarya*, with dominant species including *S. breviflora*, *Artemisia frigida* and *Cleistogenes songorica*. Main companion species include *Convolvulus ammannii*, *Heteropappus altaicus*, etc. According to the classification system of the Food and Agriculture Organization of the United Nations, the soil is light chestnut soil with sandy loam texture, rich in potassium but low in nitrogen, phosphorus, and organic matter content.

The experiment initiated in May 2006 adopted a 2 × 2 factorial split-plot design, with warming as the main treatment (control/warming) and nitrogen addition as the sub-treatment (no nitrogen/nitrogen addition). Four treatments were set up: Control (C), Warming (W), Nitrogen Addition (N), and Warming + Nitrogen Addition (W + N), each with 6 replicates, totaling 144 m^2. Warming was achieved using 2000 W infrared radiators suspended 2.25 m above the center of the plots, with an average annual surface temperature increase of 1.3 °C. Dummy radiators of the same size were installed in control plots to eliminate errors. Nitrogen was applied as ammonium nitrate (10 $g \cdot m^{-2} \cdot yr^{-1}$) once a year from late June to early July, dissolved in water and sprayed evenly on cloudy days or during early morning or evening to prevent volatilization. Non-nitrogen plots received equivalent water to maintain consistent moisture levels (Figure 1).

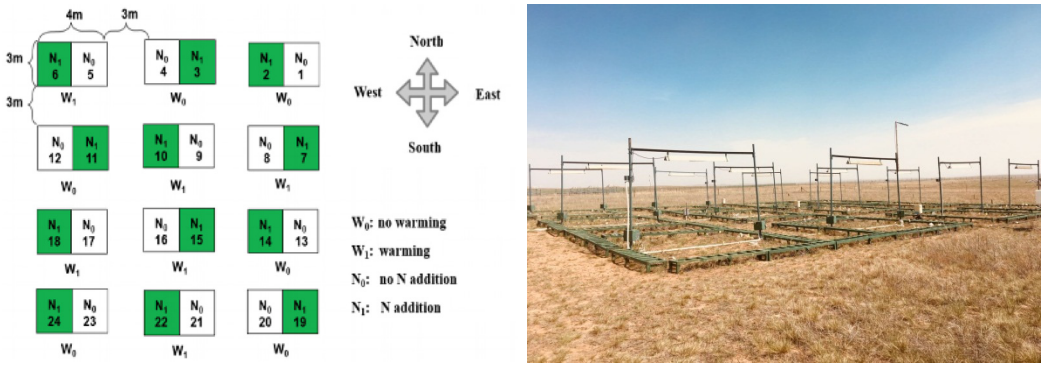


Figure 1. The general view of experimental site and experimental layout.

2.2. Soil Aggregate Size Classification and Other Soil Physicochemical Attributes

At the end of August 2018 and 2019, We used a shovel to remove approximately 50 g of undisturbed soil from a 0–10 cm soil layer, putting the removed soil in a disposable lunch box for preservation and took it back to the laboratory for aggregate screening. After aggregate screening, a part of the soil sample was stored in a ziplock bag and quickly brought back to the laboratory for low-temperature storage and stored in a refrigerator at 4 °C for determination of soil enzyme activity. The other part of the sample was air-dried to determine the physical and chemical indicators in the soil. To minimize the disruption in microbial communities and activities, soil aggregates were isolated by a modified dry-sieving method. When the soil moisture content was 10–15%, it is suitable to break the soil along the natural texture with a small external mechanical force. Soil was placed on a Retsch AS200 (Verder Shanghai Instruments and Equipment Co., Ltd., Shanghai, China) instrument set sieve. The soil sample was vibrated with an amplitude for 2 min, and the soil samples were divided into the following three aggregate sizes: large aggregates (>2000 μm), small aggregates (250–2000 μm) and micro-aggregates (<250 μm). After screening, the aggregate content of >2000 μm particle size accounts for about 23% of the total aggregate, and the content of 250–2000 μm particle size aggregates accounts for about 40% of the total aggregates, and the content of <250 μm particle size aggregates accounts for about 37% of the total aggregates [27].

The pH and conductivity of soil aggregates were measured with a PHS-3G digital pH meter (Precision and Scientific Crop., Shanghai, China). About 10 g of air-dried soil was weighed out and placed in a 50 mL Erlenmeyer flask and measured soil pH with a graduated cylinder. We poured 25 mL of deionized water into an Erlenmeyer flask, stirred it evenly, and let it stand for half an hour for determination. The pH-water-soil ratio was 1:2.5. The soil water content (SWC) was measured using the aluminum box-drying method. The SOC content in the soil aggregates was measured by potassium dichromate-external heating method; the TN content was measured with an element analyzer after the soil had been finely ground by a ball mill; and the TP was measured by the sodium hydroxide melting method [32,33]. All reagents used for the determination of indicators were from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.3. Determination of Soil Enzyme Activity

For Urease activity, the indophenol blue colorimetry is used. A 2 g sample of air-dried soil passing a 1 mm sieve is weighed into a 50 mL conical flask, treated with 1 mL of toluene to moisten the soil, and allowed to stand for 15 min. Then, 10 mL of urea solution (10%) (AR, Sinopharm, Shanghai, China) and 20 mL of citrate buffer (0.1 mol/L, pH 6.7) (AR, Sinopharm, Shanghai, China) are added, thoroughly mixed, and incubated at 37 °C for 24 h. After incubation, the mixture is diluted to the mark with warm water (~38 °C), shaken well, and filtered. One milliliter of the filtrate is transferred to a 50 mL volumetric flask, distilled

water is added and shaken, followed by the addition of 4 mL of sodium phenolate solution (0.5 mol/L) (AR, Sinopharm, Shanghai, China) and 3 mL of sodium hypochlorite solution (0.1 mol/L) (AR, Sinopharm, Shanghai, China). After shaking and standing for 20 min, the solution is diluted to the mark. The indophenol blue color is measured at 578 nm using a spectrophotometer (Techcomp, Shanghai, China). Soil-free and substrate-free controls (with water replacing soil or urea solution, respectively) are set up, and the activity is calculated using the formula: Soil Urease = (asample − asoil-free − asubstrate-free) × V × n/m, where asample is the milligrams of NH_3-N obtained from the standard curve based on the absorbance value of the sample; asoil-free is the milligrams of NH_3-N obtained from the standard curve based on the absorbance value of the soil-free control; asubstrate-free is the milligrams of NH_3-N obtained from the standard curve based on the absorbance value of the substrate-free control; V is the color development solution volume (mL); n is the aliquot multiple (leachate volume/filtrate volume); and m is the oven-dried soil weight (g) [34].

For ALP activity, the sodium phenolphthalein diphosphate colorimetry is applied. A 5 g sample of air-dried soil passing a 1 mm sieve is weighed into a 200 mL Erlenmeyer flask, treated with 2.5 mL of toluene for 15 min, followed by the addition of 20 mL of sodium phenolphthalein diphosphate solution (0.5%) (AR, Sinopharm, Shanghai, China), and incubated at 37 °C for 24 h. After incubation, 100 mL of aluminum sulfate solution (0.3%) (AR, Sinopharm, Shanghai, China) is added and filtered through phosphorus-free filter paper. Three milliliters of the filtrate is transferred to a 50 mL volumetric flask, 5 mL of boric acid buffer (0.5 mol/L, pH 9.0) (AR, Sinopharm, Shanghai, China) and 0.2 mL of chlorodibromo-p-benzoquinone imine reagent (0.5%) (AR, Sinopharm, Shanghai, China) are added for color development, diluted to the mark, and the absorbance is measured at 660 nm after 30 min. Controls are set up similarly to Urease, with the formula: Soil ALP = (asample − asoil-free − asubstrate-free) × V × n/m, where asample is phenol milligrams obtained from the standard curve based on the absorbance value of the sample; asoil-free is phenol milligrams obtained from the standard curve based on the absorbance value of the soil-free control; asubstrate-free is phenol milligrams obtained from the standard curve based on the absorbance value of the sub-strate-free control; V is volume of the color-developing solution (mL); n is the aliquot multiple (leachate volume/filtrate volume); and m is the oven-dried soil weight (g) [34].

For BG activity, the nitrophenol colorimetry is used. A 1 g sample of fresh soil passing a 1 mm sieve is weighed into a 100 mL conical flask, 0.25 mL of toluene (AR, Sinopharm, Shanghai, China) is added, and the flask is placed in a fume hood for 10 min. Then, 4 mL of pH 6.0 MUB (modified universal buffer, 0.1 mol/L) and 1 mL of PNPG (p-nitrophenyl-β-D-glucopyranoside) solution (0.005 mol/L) (PNPG, Biotech grade, Sinopharm, Shanghai, China) are added, mixed, and incubated at 37 °C for 1 h. After incubation, 1 mL of $CaCl_2$ solution (0.5 mol/L) (AR, Sinopharm, Shanghai, China) and 4 mL of 0.1 mol/L Tris buffer (pH 12.0) (tris(hydroxymethyl)aminomethane, AR, Sinopharm, Shanghai, China) are added, shaken, filtered, and the absorbance is measured at 400 nm. Soil-free (water instead of soil) and substrate-free (water instead of PNPG) controls are included, with the formula: Soil BG = CV/dwt, where C is the p-nitrophenol content (μg/mL), V is the solution volume, and dwt is the oven-dried soil mass (g) [34].

For DHA activity, the TTC reduction method is employed. A 2 g sample of fresh soil passing a 2 mm sieve is weighed into a conical flask, 2 mL of TTC solution (1%) (AR, Sinopharm, Shanghai, China) and 2 mL of glucose solution (1%) (AR, Sinopharm, Shanghai, China) are added, incubated at 37 °C for 24 h, then 20 mL of methanol is added and shaken on a shaker for 1 h. After filtration, the absorbance of the filtrate is measured at 485 nm using a soil-free control. The activity is calculated as: Soil DHA = C × V/dwt, where C is the TPF (triphenylformazan) concentration in the filtrate, V is the filtrate volume, and dwt is the oven-dried soil mass (g) [34].

2.4. Statistical Analysis

The main effects and interactions (W × N, W × S, N × S, W × N × S) of Warming (W), Nitrogen addition (N), and Aggregate Particle Size (S) were tested by three-way ANOVA. After confirming that the main effect is significant, two factor analysis of variance was used to further analyze the specific interaction, and the least significant difference (LSD) test was used for pairwise comparison. Then correlation analysis was used to determine the correlation between soil aggregate physical and chemical properties and enzyme activity. Sigmaplot 14.0 was used to plot and statistical significance was determined at $p < 0.05$. Then correlation analysis was used to determine the correlation between soil aggregate physical and chemical properties and enzyme activity.

3. Results

3.1. Soil pH, SWC, SOC, TN and TP Values Among Soil Aggregates

The three-way ANOVA showed that the size of soil aggregates exerted a significant influence on nearly all the indicators ($p < 0.05$), while the effects of warming and nitrogen addition were relatively minor. The interaction between warming and nitrogen addition had no significant effect on all indicators, indicating that the impacts of warming and nitrogen addition on soil properties were relatively independent (Table 1).

In 2018, Different treatments showed significant differences in pH values among soil aggregate sizes. For large aggregates, the pH was highest in the warming treatments, while the lowest pH was observed in the nitrogen addition treatment. Nitrogen addition significantly decreased the pH of small aggregates ($p < 0.05$). Soil aggregate size had a significant impact on pH, with micro-aggregates showing the highest pH values across all treatments. In 2019, Soil aggregate size remained a significant factor for pH ($p < 0.0001$), with micro-aggregates having the lowest pH. The effect of warming on pH diminished, and the impact of nitrogen addition on pH became less pronounced across different aggregate sizes (Table 2). Soil aggregate size significantly affected SWC ($p < 0.0001$), with micro-aggregates and small aggregates having higher water content than large aggregates. Nitrogen addition significantly increased the water content of micro-aggregates and small aggregates ($p < 0.05$), likely due to enhanced soil microbial activity or improved soil structure from nitrogen fertilization. Warming significantly decreased SWC in 2019 ($p < 0.05$), indicating that warming may reduce soil moisture through increased evaporation (Figure 2a).

Table 1. Results (*F*-value) of three-way ANOVAs with a split-plot design on the effect of warming (W), nitrogen addition (N) and soil aggregate sizes (S) on Soil pH, SWC, SOC, TN and TP values.

Years	Factors	pH	SWC	SOC	TN	TP
		***F* Value**	***F* Value**	***F* Value**	***F* Value**	***F* Value**
2018	W	2.02	2.26	11.66 *	8.01 *	1.32
	N	18.75 **	0.33	0.61	1.34	0.05
	S	3.59 *	26.52 ***	2.12	9.06	0.67
	W×N	0.03	0.94	0.19	0.01	0.02
	W×S	1.95	1.69	0.08	1.33	0.45
	N×S	0.83	0.11	1.30	1.41	0.18
	W×N×S	0.82	1.12	0.18	1.74	0.27
2019	W	0.63	7.61 *	5.05 *	2.21	0.09
	N	4.04	1.8	0.48	1.40	0.14
	S	26.76 ***	4.18 *	3.67 *	4.79 *	2.04
	W×N	0.06	0.18	2.53	0.66	0.00
	W×S	1.11	0.65	0.14	1.29	0.21
	N×S	0.89	1.39	0.56	0.09	0.71
	W×N×S	0.31	1.48	0.98	0.33	0.30

Note: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$.

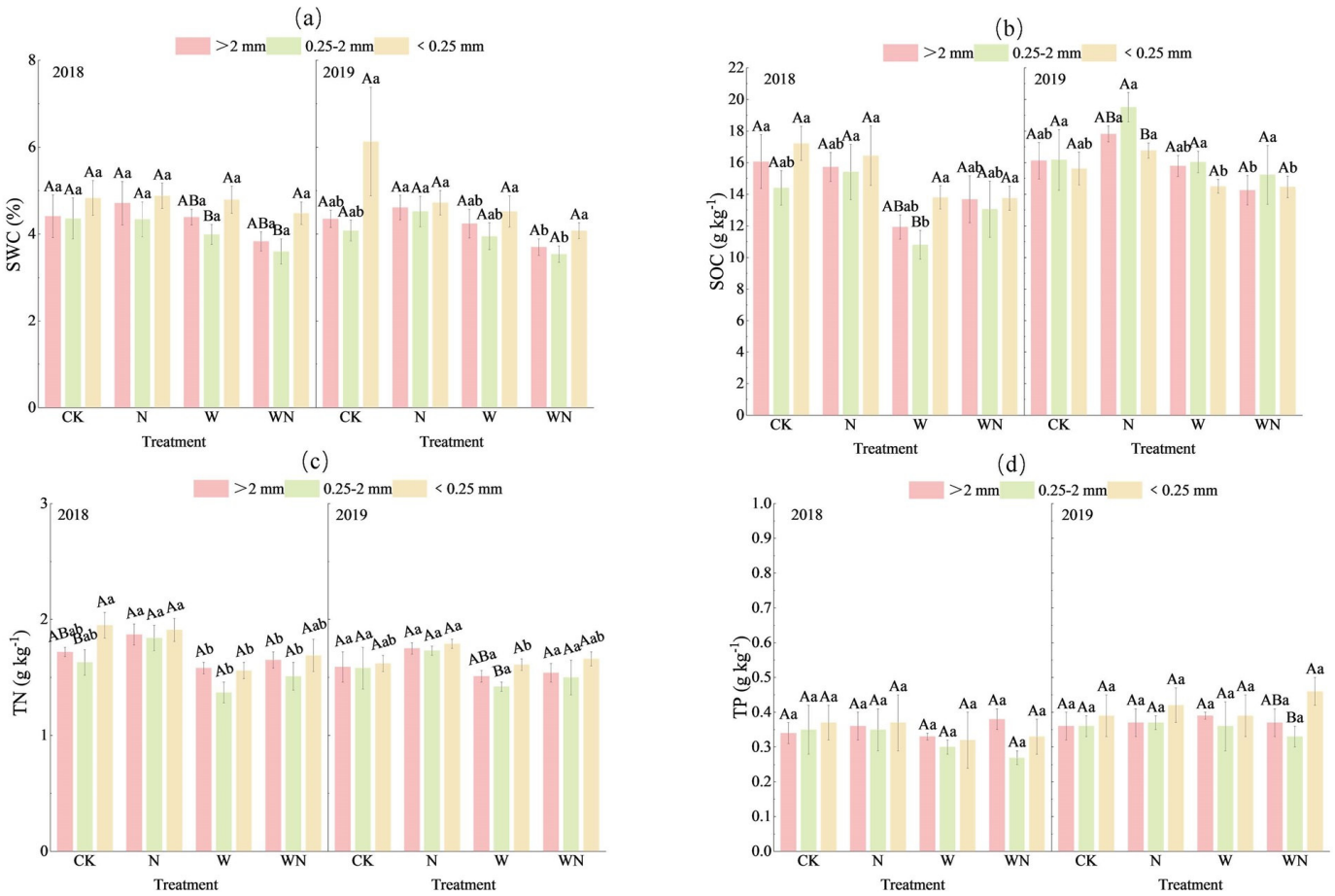


Figure 2. SWC, SOC, TN and TP in different soil aggregate sizes under each treatment in 2018–2019. Note 1: SWC, SOC, TN, and TP represent soil water content, soil organic carbon, total nitrogen, and total phosphorus, respectively. Note 2: (**a**) SWC in different soil aggregate sizes under each treatment in 2018–2019; (**b**) SOC content in different soil aggregate sizes under each treatment in 2018–2019; (**c**) TN content in different soil aggregate sizes under each treatment in 2018–2019; (**d**) TP content in different soil aggregate sizes under each treatment in 2018–2019. Note 3: The data in this figure are presented as mean ± standard deviation. Different capital letters indicate significant differences among various soil aggregates under identical treatments ($p < 0.05$), while different lowercase letters denote significant differences among the same soil aggregates across distinct treatments ($p < 0.05$).

In 2018, SOC content was highest in micro-aggregates and lowest in small aggregates. Warming led to a notable reduction in the SOC content within small-sized soil aggregates ($p < 0.05$). In 2019, SOC content was highest in small aggregates and lowest in micro-aggregates. Warming had no significant effect on SOC content, and nitrogen addition also had no significant effect on SOC content ($p > 0.05$) (Figure 2b). The distribution trend of TN in different aggregate sizes was: micro-aggregates > large aggregates > small aggregates. Warming significantly decreased TN content in micro-aggregates in 2018 ($p < 0.05$), but did not exert a significant impact on aggregates of other sizes. Nitrogen addition had no significant effect on TN content ($p > 0.05$) (Figure 2c). The distribution trend of TP in different aggregate sizes was similar to that of SOC and TN: small aggregates < large aggregates < micro-aggregates. Neither warming nor nitrogen addition had a significant effect on TP content ($p > 0.05$), suggesting that changes in TP content may be related to plant residue inputs and the stability of mineral-bound phosphorus (Figure 2d).

Table 2. Soil pH in different soil aggregate sizes under each treatment in 2018 and 2019.

Years	Aggregate Sizes (μm)	pH			
		CK	N	W	W + N
2018	>2000	7.67 ± 0.04 ^{Aa}	7.65 ± 0.02 ^{Aa}	7.73 ± 0.03 ^{Aa}	7.36 ± 0.24 ^{Aa}
	250–2000	7.52 ± 0.01 ^{Bab}	7.12 ± 0.18 ^{Ac}	7.71 ± 0.02 ^{Aa}	7.27 ± 0.14 ^{Abc}
	<250	7.65 ± 0.02 ^{Aab}	7.06 ± 0.21 ^{Ab}	7.76 ± 0.01 ^{Aa}	7.21 ± 0.24 ^{Aab}
2019	>2000	8.40 ± 0.22 ^{Aa}	8.00 ± 0.29 ^{Aa}	8.41 ± 0.20 ^{Aa}	8.10 ± 0.36 ^{Aa}
	250–2000	8.30 ± 0.26 ^{ABa}	7.63 ± 0.20 ^{Ba}	8.39 ± 0.29 ^{Aa}	7.89 ± 0.43 ^{Aa}
	<250	7.76 ± 0.07 ^{Ba}	7.46 ± 0.11 ^{Aa}	8.00 ± 0.11 ^{Aa}	7.53 ± 0.39 ^{Aa}

Note: The data in this figure are presented as mean ± standard deviation. Different capital letters indicate significant differences among various soil aggregates under identical treatments ($p < 0.05$), while different lowercase letters denote significant differences among the same soil aggregates across distinct treatments ($p < 0.05$).

3.2. Response of Enzyme Activities in Different Soil Aggregate Classes to Warming and Nitrogen Addition

Results of the three-way ANOVA revealed significant effects of warming on urease activity in both 2018 ($p < 0.05$) and 2019 ($p < 0.01$), whereas nitrogen addition significantly influenced urease activity only in 2018 ($p < 0.01$) but not in 2019 ($p > 0.05$). Aggregate size significantly affected Urease activity in both years ($p < 0.01$) and BG activity in 2018 ($p < 0.0001$). Nitrogen addition significantly impacted BG activity in both 2018 ($p < 0.01$) and 2019 ($p < 0.05$), while warming and its interaction with nitrogen had no significant effect on this enzyme. For ALP activity, warming and aggregate size had significant effects in 2018 and 2019, respectively ($p < 0.05$), but nitrogen addition did not. In 2019, aggregate size significantly influenced DHA activity ($p < 0.05$), whereas warming and nitrogen addition had no significant impact (Table 3).

Table 3. Results (*F*-value) of three-way ANOVAs with a split-plot design on the effect of warming (W), nitrogen addition (N) and soil aggregate sizes (S) on soil Urease, BG, ALP and DHA activities.

Years	Factors	Urease *F* Value	BG *F* Value	ALP *F* Value	DHA *F* Value
2018	W	5.30 *	1.95	5.00 *	1.05
	N	10.84 **	8.93 **	0.02	2.74
	S	5.28 **	61.92 ***	2.91	1.53
	W×N	2.57	1.03	0.22	1.28
	W×S	0.78	2.2	2.31	1.84
	N×S	0.52	2.48	0.23	0.25
	W×N×S	1.61	2.51	0.05	0.1
2019	W	14.57 **	4.33	7.14	0.37
	N	0.94	4.78 *	0.6	0.28
	S	7.13 **	1.69	12.56 *	5.16 *
	W×N	0.79	0.82	0.06	1.15
	W×S	2.49	0.59	1.64	0.02
	N×S	3.92 *	0.81	0.12	0.12
	W×N×S	0.56	1.46	0.21	0.11

Note: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$.

In 2018, warming, nitrogen addition, and warming + nitrogen addition treatments all significantly reduced urease activity in small aggregates ($p < 0.05$). Nitrogen addition and warming + nitrogen addition treatments significantly reduced Urease activity in micro-aggregates ($p < 0.05$), which decreased by 34.32% and 30.03%, respectively. In 2019, warming and warming + nitrogen addition significantly reduced micro-aggregate Urease activity ($p < 0.05$). Urease activity in small aggregates under nitrogen addition

was significantly lower than that in micro-aggregates ($p < 0.05$). Throughout the experiment, micro-aggregates consistently exhibited the highest urease activity among different aggregate sizes (Figure 3a).

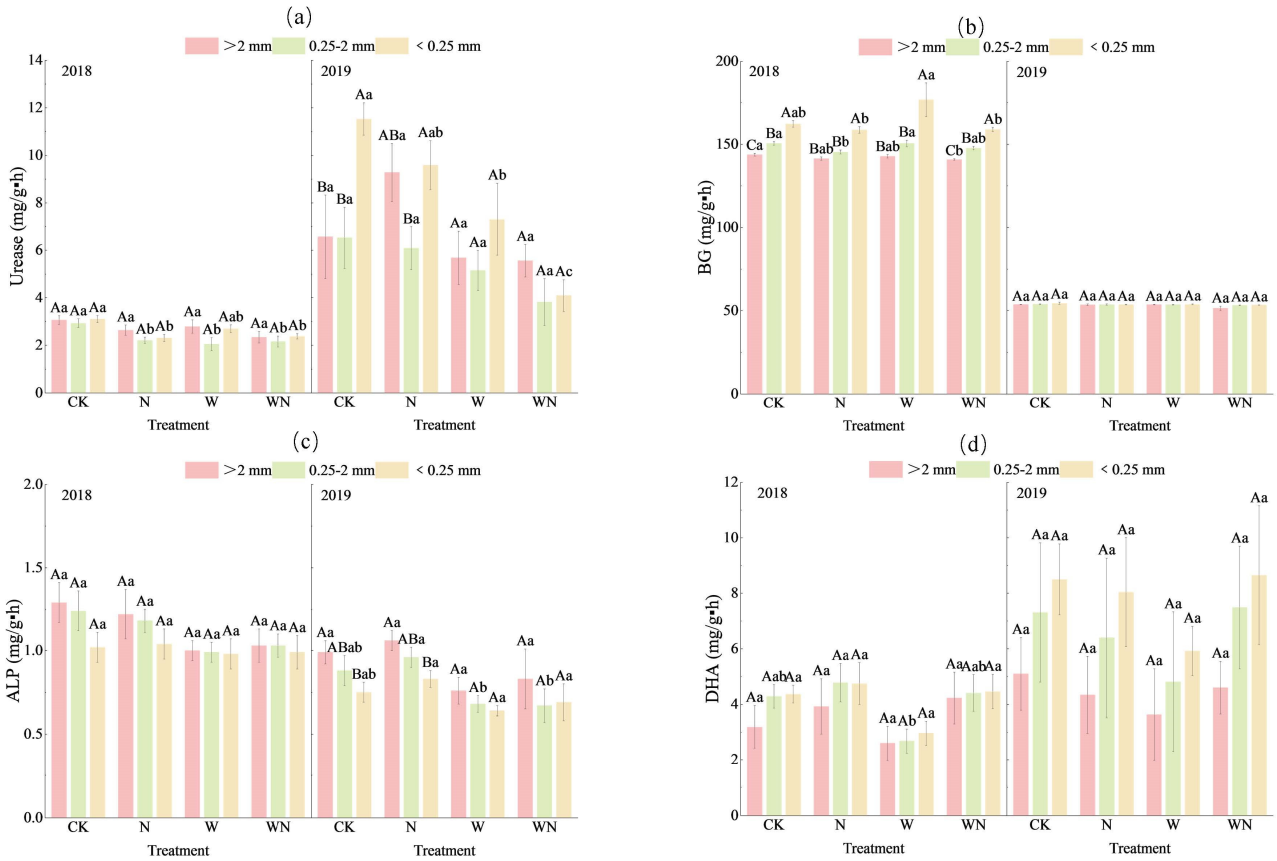


Figure 3. Soil Urease, BG, ALP and DHA activities in different soil aggregate sizes under each treatment in 2018–2019. Note 1: BG, ALP and DHA represent β-glucosidase, Alkaline Phosphatase and Dehydrogenase activities, respectively. Note 2: (**a**) Soil Urease activities in different soil aggregate sizes under each treatment in 2018–2019; (**b**) Soil BG activities in different soil aggregate sizes under each treatment in 2018–2019; (**c**) Soil ALP activities in different soil aggregate sizes under each treatment in 2018–2019; (**d**) Soil DHA activities in different soil aggregate sizes under each treatment in 2018–2019. Note 3: The data in this figure are presented as mean ± standard deviation. Different capital letters indicate significant differences among various soil aggregates under identical treatments ($p < 0.05$), while different lowercase letters denote significant differences among the same soil aggregates across distinct treatments ($p < 0.05$).

In 2018, under conditions of warming and nitrogen addition, BG activity in micro-aggregates showed a significant increase compared to in small and large aggregates ($p < 0.05$), and there were significant differences in activity among all aggregate sizes under control and warming + nitrogen addition treatments ($p < 0.05$). Compared to the control, nitrogen addition reduced BG activity in small aggregates by 3.61%, and warming combined with nitrogen addition decreased activity in large aggregates by 2.03%. In 2019, the effects of warming, nitrogen addition, and warming + nitrogen addition treatments on BG activity across different aggregate sizes were not significant ($p > 0.05$). Over the two-year experiment, BG activity gradually decreased with increasing aggregate size (Figure 3b).

Figure 3c reveals that in 2018, ALP activity across treatments followed a trend of decreasing with increasing aggregate size (micro-aggregate < small aggregate < large aggregate), with non-significant effects ($p > 0.05$). In 2019, nitrogen addition significantly reduced macroaggregate and micro-aggregate activity by 26.62%. The order of activity

remained consistent across treatments, except under warming + nitrogen addition where large aggregates showed the highest activity. Despite changes, effects were non-significant ($p > 0.05$) (Figure 3c).

Through two years of experiments, it was found that consistent with the response of the whole soil, nitrogen addition increased the activity of DHA in aggregate, while warming decreased the activity of DHA in the aggregate, but the effect was not significant ($p > 0.05$). Under the treatments of warming, nitrogen addition and warming + nitrogen addition, the distribution trend of DHA activity in the aggregate all followed the order: large aggregate < small aggregate < micro-aggregate (Figure 3d).

3.3. Correlation Analysis Between Enzyme Activities Within Soil Aggregates and Physicochemical Properties

The analysis of soil enzyme activities within different aggregate sizes during the experimental period revealed distinct correlations with soil physicochemical properties. In large aggregates, Urease activity exhibited a significant negative correlation with soil TP in 2018. DHA activity in 2018 was negatively correlated with soil pH and SWC, while it positively correlated with TN and SOC. In 2019, ALP activity showed a negative correlation with soil pH and a positive correlation with SOC. BG activity was positively correlated with both soil pH and SWC in 2018, with the correlation remaining significant only with SWC in 2019 (Table 4).

Table 4. The correlation between physicochemical properties and enzyme activity of large aggregates in 2018 and 2019.

Years		pH	SWC	TC	TN	SOC	Urease	DHA	ALP	BG
2018	pH	1	0.28	−0.18	−0.35	−0.3	0.17	−0.75 ***	0.11	0.45 *
	SWC	0.28	1	−0.71	−0.13	0.17	0.26	−0.54 **	0.19	0.43 *
	TC	−0.18	−0.71	1	0.92 ***	0.27	0.025	0.26	0.26	0.094
	TN	−0.35	−0.13	0.92 ***	1	0.34	0.022	0.42 *	0.2	−0.054
	SOC	−0.3	0.17	0.27	0.34	1	0.34	0.42 *	0.19	0.056
	Urease	0.17	0.26	0.025	0.022	0.34	1	−0.19	0.13	0.2
	DHA	−0.75 ***	−0.54 **	0.26	0.42 *	0.42 *	−0.19	1	−0.02	−0.38
	ALP	0.11	0.19	0.26	0.2	0.19	0.13	−0.02	1	0.48 *
	BG	0.45 *	0.43 *	0.094	−0.054	0.056	0.2	−0.38	0.48 *	1
2019	pH	1	0.13	−0.31	0.0047	−0.4	−0.13	−0.15	−0.44 *	0.17
	SWC	0.13	1	0.17	−0.28	0.38	−0.2	0.07	0.29	0.43 *
	TC	−0.31	0.17	1	0.41 *	0.8 ***	0.26	−0.044	0.71 ***	0.24
	TN	0.0047	−0.28	0.41 *	1	0.22	0.19	0.063	0.31	0.14
	SOC	−0.4	0.38	0.8 ***	0.22	1	0.19	0.047	0.62 **	0.26
	Urease	−0.13	−0.2	0.26	0.19	0.19	1	−0.041	0.014	−0.36
	DHA	−0.15	0.07	−0.044	0.063	0.047	−0.041	1	−0.087	0.11
	ALP	−0.44 *	0.29	0.71 ***	0.31	0.62 **	0.014	−0.087	1	0.17
	BG	0.17	0.43 *	0.24	0.14	0.26	−0.36	0.11	0.17	1

Note: *, **, *** represent $p < 0.5$, $p < 0.1$, $p < 0.001$, respectively.

For small aggregates, a significant positive correlation was observed between Urease activity and soil pH in 2019. DHA activity in 2018 was negatively correlated with soil pH and positively with TN and SOC. ALP activity was positively correlated with SWC in 2018 and negatively with soil pH in 2019, while it positively correlated with SWC and organic carbon. BG activity exhibited a significant positive correlation with soil pH exclusively in 2018 (Table 5).

Table 5. The correlation between physicochemical properties and enzyme activity of small aggregates in 2018 and 2019.

Years		pH	SWC	TC	TN	SOC	Urease	DHA	ALP	BG
2018	pH	1	0.28	−0.75 ***	−0.62 **	−0.78 ***	−0.071	−0.63 **	0.037	0.56 **
	SWC	0.28	1	−0.022	0.12	0.015	0.15	−0.13	0.52 *	−0.041
	TC	−0.75 ***	−0.022	1	0.96 ***	0.97 ***	0.17	0.53 **	0.15	−0.28
	TN	−0.62 **	0.12	0.96 ***	1	0.95 ***	0.23	0.43 *	0.29	−0.14
	SOC	−0.78 ***	0.015	0.97 ***	0.95 ***	1	0.21	0.50 *	0.21	−0.29
	Urease	−0.071	0.15	0.17	0.23	0.21	1	0.33	0.53 **	0.33
	DHA	−0.63 **	−0.13	0.53 **	0.43 *	0.50 *	0.33	1	0.21	−0.41
	ALP	0.037	0.52 *	0.15	0.29	0.21	0.53 **	0.21	1	0.17
	BG	0.56 **	−0.041	−0.28	−0.14	−0.29	0.33	−0.41	0.17	1
2019	pH	1	0.15	−0.65 ***	−0.36	−0.63 ***	0.56 **	−0.072	−0.57 **	0.40 *
	SWC	0.15	1	0.29	0.022	0.33	0.19	0.053	0.49*	−0.035
	TC	−0.65 ***	0.29	1	0.44 *	0.86 ***	−0.13	0.19	0.76 ***	−0.34
	TN	−0.36	0.022	0.44 *	1	0.41 *	−0.014	−0.012	0.37	0.041
	SOC	−0.63 ***	0.33	0.86 ***	0.41 *	1	−0.21	−0.024	0.68 ***	−0.28
	Urease	0.56 **	0.19	−0.13	−0.014	−0.21	1	−0.18	−0.11	−0.0038
	DHA	−0.072	0.053	0.19	−0.012	−0.024	−0.18	1	0.036	0.034
	ALP	−0.57 **	0.49 *	0.76 ***	0.37	0.68 ***	−0.11	0.036	1	−0.26
	BG	0.40 *	−0.035	−0.34	0.041	−0.28	−0.0038	0.034	−0.26	1

Note: *, **, *** represent $p < 0.5$, $p < 0.1$, $p < 0.001$, respectively.

Micro-aggregates displayed correlations similar to those observed in large aggregates, with no significant relationship between Urease activity and soil physicochemical properties. DHA activity in 2018 was negatively correlated with soil pH and SWC, and positively with TN. In 2019, ALP activity displayed a negative correlation with soil pH and a positive correlation with TC. BG activity showed a positive correlation with SWC in 2019 (Table 6).

Table 6. The correlation between physicochemical properties and enzyme activity of micro-aggregates in 2018 and 2019.

Years		pH	SWC	TC	TN	SOC	Urease	DHA	ALP	BG
2018	pH	1	0.19	−0.31	−0.46 *	−0.28	0.37	−0.64 **	0.28	0.36
	SWC	0.19	1	0.056	0.082	−0.38	−0.085	−0.50 *	0.33	−0.2
	TC	−0.31	0.056	1	0.94 ***	0.29	0.0086	0.34	−0.062	−0.3
	TN	−0.46 *	0.082	0.94 ***	1	0.33	0.0093	0.43 *	−0.14	−0.35
	SOC	−0.28	−0.38	0.29	0.33	1	0.24	0.04	−0.23	−0.024
	Urease	0.37	−0.085	0.0086	0.0093	0.24	1	0.024	0.045	0.0047
	DHA	−0.64 **	−0.50 *	0.34	0.43 *	0.04	0.024	1	−0.32	−0.33
	ALP	0.28	0.33	−0.062	−0.14	−0.23	0.045	−0.32	1	0.18
	BG	0.36	−0.2	−0.3	−0.35	−0.024	0.0047	−0.33	0.18	1
2019	pH	1	0.14	−0.67 ***	−0.29	−0.37	0.28	0.23	−0.67 ***	0.40
	SWC	0.14	1	−0.25	−0.034	−0.047	0.28	0.027	0.083	0.69 ***
	TC	−0.67 ***	−0.25	1	0.28	0.76 ***	−0.074	−0.048	0.47 *	−0.5 *
	TN	−0.29	−0.034	0.28	1	0.32	−0.09	0.026	0.17	−0.16
	SOC	−0.37	−0.047	0.76 ***	0.32	1	0.33	0.12	0.35	−0.34
	Urease	0.28	0.28	−0.074	−0.09	0.33	1	−0.11	0.14	0.15
	DHA	0.23	0.027	−0.048	0.026	0.12	−0.11	1	−0.22	−0.041
	ALP	−0.67 ***	0.083	0.47 *	0.17	0.35	0.14	−0.22	1	−0.25
	BG	0.40	0.69 ***	−0.5 *	−0.16	−0.34	0.15	−0.041	−0.25	1

Note: *, **, *** represent $p < 0.5$, $p < 0.1$, $p < 0.001$, respectively.

These results suggest that the responsiveness of soil enzyme activities to physicochemical properties is size-dependent and highlights the complexity of these interactions within different soil aggregate fractions.

4. Discussion

4.1. Effects of Warming and Nitrogen Addition on the Content of C, N and P in Soil Aggregates

As a huge carbon pool, SOC is crucial for modulating the global carbon cycle and influencing global warming dynamics [12,35]. However, there are still great uncertainties in existing studies on the impact of warming on SOC dynamics. It is generally thought that the natural heating gradient mediates the SOC decomposition reaction [36].

In the two years of our experiment, warming had different effects on the SOC content in soil aggregates. In 2018, the SOC content in micro-aggregates (<250 μm) was the highest, but in 2019 the SOC content in small aggregates (250–2000 μm) was the highest. One plausible mechanism for the elevated SOC in micro-aggregates under warming conditions may be attributed to the increased proportion of macroaggregates [37]. The distribution of SOC in soil aggregates may be affected by many factors, including vegetation and soil characteristics [38], so further research is needed. The effect of warming on the organic carbon content of soil aggregates is not significant, which is consistent with previous studies [39]. This may be attributed to the balance between carbon input in litter and carbon output during the process of mineralization under warming conditions. As previously mentioned, dry soil will increase the mineralization of the SOC in the soil [40], but it reduces the decomposition of fresh organic matter returned to the soil [41]. Warming led to a decreasing trend of TN content in soil aggregates, but only significantly reduced the total nitrogen content of micro-aggregates in 2018 and had no significant effect on the TN content of large aggregates or small aggregates, or on the size of each aggregate in 2019. The distribution trend of TN and TP in the aggregate was the same as for SOC. Compared with the control treatment, warming had no significant effects on SOC, TN or TP contents in soil aggregates of different sizes. This is consistent with the results of a study conducted in alpine meadows [42]. In previous research [43,44], changes in biotic factors (such as vegetation biomass and microbial activity) and abiotic factors (such as soil moisture and altitude gradient) were considered as the causes for significant changes in soil nutrients. However, some studies have shown that warming significantly increased the contents of SOC and TN in meadow steppes but had no significant effect on TP content [45]. Therefore, it is evident that the current understanding of the impacts of warming on soil physical and chemical properties still warrants further research and discussion.

In addition, the impact of nitrogen addition on SOC content is currently unclear. Previous studies have shown that nitrogen addition can alter the chemical properties of dead branches and leaves, such as aromatic properties, carbon-to-nitrogen ratio, and saturated as well as carbonyl carbon contents in above- and belowground biomass [46,47], and litter decomposition [48], consequently changing soil C inputs. Moreover, N enrichment may regulate the composition and decrease the activity of soil microbial communities, resulting in a reduction in soil C emissions [49–51]. In this study, in 2018, the SOC content in the micro-aggregates was the highest, and the SOC content in the small aggregates was the lowest. This is the same as previous research results [52,53]. The possible reasons for this phenomenon may be that physical protection by micro-aggregates dominated SOC accumulation in 2018, with more active decomposition of small aggregates; while in 2019, small aggregates might have become the main storage units for SOC due to enhanced structural stability, increased carbon input, or reduced decomposition [37,54]. This phenomenon also reflects the complexity of desert steppe soil responses to climate change, which requires long-term monitoring for verification. However, in 2019 it was found that the SOC content was the highest in small aggregates. Similar to the warming treatment, the distribution trend of TN and TP in aggregates was the same as for SOC. In the three aggregate size classes, the distribution of SOC, TN and TP followed the same order: micro-aggregates > large aggregates > small aggregates. The nutrient advantages of large aggregates originate

from their physical structure's capacity to capture fresh organic matter and the active microbial metabolic cycles [13,55]. These mechanisms collectively lead to large aggregates becoming "hotspots" for soil nutrients (particularly active C, N, and P), while small aggregates tend to store nutrients in a more stable state [56]. The higher carbon and nitrogen content in micro-aggregates is mainly due to stronger physical protection [53], and in semi-arid areas this protection will be stronger due to drought. Previous studies have shown that the TC and TN content of large aggregates and small aggregates is higher than that of micro-aggregates, which is consistent with the results of this study [57]. The results of the Rothamsted C model related to soil organic matter consistently, and this will help enhance the stability of organic matter in the aggregates [58]. The TP content in large aggregates was higher than in small aggregates, mainly due to the higher input of plant residues in large aggregates [59]. The main reason for the higher TP content in micro-aggregates is primarily closely related to the adsorption of clay minerals (such as illite) and iron and aluminum oxides [60] and better protection of organic matter (the diffusion of moisture and oxygen is limited) [61] and high stability of organic matter (the ability of soil organic carbon to resist decomposition and maintain long-term storage in the ecosystem) [31].

4.2. Effects of Warming and Nitrogen Addition on Enzyme Activities in Soil Aggregates

Soil extracellular enzymes come from the secretion of soil microorganisms, plant roots, or the release of cell death and lysis, which affect the conversion cycle of soil nutrients and can reflect the dynamic changes of soil nutrients [62]. Enzymatic reactions in soil depend on temperature. Warming allows the reactants to obtain more activation energy, thereby increasing enzyme activity. Thermodynamic theory predicts that enzyme activity parameters will also increase with warming [63]. This study found that warming increases the activity of BG in soil aggregates, which is consistent with previous studies [64]. This is attributed to the fact that elevated temperature under sufficient moisture conditions promotes microbial migration, nutrient movement, and enzyme-substrate binding in soil solution [65]. Soil pH is considered to be the main control factor for microbial activity enzyme kinetics [41,66] and microbial diversity [67]. So, nitrogen addition will cause soil acidification [68], which will affect the activity and composition of microorganisms [69]. Soil aggregates have an important influence on the distribution of soil enzyme activity [70]. Previous studies have shown that nitrogen addition has a promoting effect on carbon-acquiring enzymes (such as BG), phosphorus-acquiring enzymes (such as ALP), and hydrolytic enzymes [71]. Our results showed that nitrogen addition significantly reduced the activities of Urease, ALP, and BG in soil aggregates (Figure 3). This contradicts our hypothesis. The reason for this phenomenon may be that nitrogen input alleviates the limitation of microorganisms on nitrogen, thus reducing the demand for enzymes involved in the nitrogen cycle [72]. In addition, nitrogen-induced soil acidification (Table 1) may inhibit enzyme stability or microbial activity, especially the activity of pH-sensitive enzymes such as Urease. However, a study using urea showed that nitrogen addition did not affect soil aggregate enzyme activity, which may be caused by different types of nitrogen addition [25]. Therefore, the response of soil enzyme activity to nitrogen deposition is multifaceted, and it is necessary to comprehensively consider soil nutrient status, microbial conditions, form of nitrogen addition, plant community composition, and grassland types [10].

In this study, ALP activity in aggregates gradually increased with increasing aggregate particle size. The Urease activity was the highest in micro-aggregates and the lowest in small aggregates. The activity of BG and DHA increased with decreasing aggregate size. Urease, BG, and DHA had the highest enzymatic activity in aggregates with a particle size of <0.25 mm (Figure 3). This supports our hypothesis. The reason for this phenomenon may be that micro-aggregates have greater physical protection effect on

organic matter and microbial community [73]. Some studies have shown that the increase of enzyme activity related to carbon decomposition with the decrease of aggregate size may be due to the higher concentration of organic carbon in micro-aggregates than that in large aggregates [74,75], which is consistent with our findings in this study. The activities of BG and DHA were the highest in micro-aggregates and the lowest in large aggregates. For micro-aggregates, the high BG activity was mainly due to the higher proportion of rapidly growing microbial groups in the aggregate in the whole microbial community [27]. It can be seen that the influence of enzyme activity is restricted by many factors, such as climate or soil, and further research is needed [76].

5. Conclusions

This study was conducted in a desert steppe dominated by *Stipa breviflora*, *Artemisia frigida*, and *Cleistogenes songorica*, with light chestnut soil (sandy loam) characterized by low N, P, and SOC contents. Results from a long-term warming and nitrogen addition experiment showed that warming significantly decreased TN content in micro-aggregates and SOC content in small aggregates in 2018. Nitrogen addition significantly reduced the pH of small aggregates, while both treatments had no significant effect on TP. Warming decreased the activities of Urease and ALP, with more pronounced effects in small and micro-aggregates. Nitrogen addition significantly reduced the activities of Urease, ALP, and BG, primarily in micro-aggregates. DHA showed a non-significant decrease under warming. The distribution of enzyme activities was regulated by aggregate size: Urease and BG activities were highest in micro-aggregates, while ALP activity increased with increasing aggregate size. The correlations with soil physicochemical properties varied across years. The interactive effects of warming and nitrogen addition were not simply additive, and interannual climatic variations had a significant impact on soil responses. These findings provide insights into the differential response mechanisms of soil aggregate C, N, P pools, and enzyme activities to global change in semiarid grassland ecosystems.

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